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EFFECTS OF CATECHOLAMINES AND CYCLIC AMP ON EXCITATION–CONTRACTION COUPLING IN ISOLATED SKELETAL MUSCLE FIBRES OF THE FROG

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SUMMARY

1. In skeletal muscle the presence of a positive inotropic effect induced by adrenaline has been a matter of controversy. If it exists, it could be due to catecholamines acting on the actomyosin system, on the sarcoplasmic reticulum (SR) Ca^{2+} pump or on the release or influx of Ca^{2+}. We investigated these possibilities by using intact, split and skinned skeletal muscle fibres. We also investigated whether adrenaline acts directly or through cyclic AMP.

2. Catecholamines produced an increase in twitch tension and in maximum rates of tension development and tension decay. The inotropic effect took 3 min to appear and 8 min to reach its maximum level. With tetanic stimulations the extra force appeared only at the beginning of the tetanus while approaching the same maximum level, and tended to disappear faster, the higher the frequency of stimulation. At 4 shocks/sec the peak twitch tension with catecholamines decreased during the first seven to ten twitches and became steady afterwards at a level that was still greater than the control.

3. Resting and action potentials showed no important changes in the presence of adrenaline that could explain the inotropic effect.

4. In split fibres the force produced with the release of Ca^{2+} from the SR by caffeine was 60–100 % larger when cyclic AMP was added to the previous loading solution. In skinned fibres adrenaline given directly to the interior of the cell produced no changes in contraction–relaxation cycles induced by fixed amounts of Ca^{2+} applied with a pipette.

5. These results strongly suggest that catecholamines through cyclic AMP stimulate the SR Ca^{2+} pump, increasing thereby the concentration of Ca^{2+} within the SR. This extra Ca^{2+} when released during subsequent activation may produce the increase in twitch tension.

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INTRODUCTION

Adrenaline is known to have a positive inotropic effect on heart muscle. Apart from the changes produced in the sarcolemmal Ca^{2+} currents (Carmeliet & Vereecke, 1969), another two intracellular mechanisms have been advanced to explain the increased contractility. These are that adrenaline could affect (*a*) the contractile proteins by increasing the phosphorylation of the actomyosin complex, which modulates the Ca^{2+} sensitivity of the actomyosin–ATPase complex (Mayer & Stull, 1971; Rubio, Bailey & Villar-Palasi, 1975), or (*b*) the phosphorylation of the membrane protein kinase of the sarcoplasmic reticulum (SR) (Kirchberger, Tada, Reptke & Katz, 1972), which would increase the Ca^{2+} uptake and loading of SR.

In skeletal muscle the results are contradictory. It has been reported that in mammalian diaphragm it is necessary to apply very large doses of adrenaline to produce an increased tension development (Goffart & Ritchie, 1952). In other mammalian skeletal muscles, the positive inotropic effect may or may not be present, depending on the type of preparation used (for a review, see Bowman & Nott, 1969).

In amphibian muscle preparations, the experimental results are few and contradictory. For example, Hutter & Loewenstein (1955) and Oota & Nagai (1977) reported a positive inotropic effect of adrenaline on curarized muscles stimulated directly; but Brown, Goffart & Vianna (1950) and Bowman & Nott (1969) found that adrenergic substances had no effect on the contractile properties of amphibian skeletal muscles. Rubio *et al.* (1975) reported that the increase in phosphorylation of the actomyosin complex found in heart preparations is not present in skeletal muscle.

Two questions arise then: (*a*) does adrenaline have a clear positive inotropic effect on frog skeletal muscle, and (*b*) if so, what mechanism is responsible for this effect? In relation to the second question, several possibilities should be considered. For example, in the heart, the prolongation of the action potential plateau and increase in inward Ca^{2+} current due to adrenaline (Carmeliet & Vereecke, 1969; Morad & Rolett, 1972) is believed to bring about the increase in tension (Wood, Heppner & Weidmann, 1969) and a similar process might occur in skeletal muscles, where an inward Ca^{2+} current has also been described (Beaty & Stefani, 1976). Secondly, the adrenaline could act directly or through other substances on either the actomyosin–ATPase complex or the SR. Since adrenaline stimulates the production of cyclic adenosine 3′:5′-monophosphate (cyclic AMP) in skeletal muscle (Rall & Sutherland, 1958; for a review see Mayer & Stull, 1971), this cyclic nucleotide may perhaps be responsible for the positive inotropic effect. The present experiments were undertaken to explore the above questions. Although the preparations used here differ from some of those used by other researchers, the results described show a clear positive inotropic effect of adrenaline on isolated skeletal muscle cells of the frog. This effect, mediated through cyclic AMP, appears to stimulate the SR Ca^{2+} pump, which leads to a larger Ca^{2+} load in the SR. Preliminary accounts of these results have been published elsewhere (Valle-Aguilera & Gonzalez-Serratos, 1976, 1977).

METHODS

The experiments were done in three different types of preparations: intact, split and skinned muscle fibres. Care was taken to ensure that all frogs used were in good condition.

Intact muscle preparations

The experimental arrangement. For the tension experiments single twitch fibres or bundles of three to four fibres were isolated from the semitendinosus muscle of the frog *Rana pipiens* (Mexican) as described previously (Gonzalez-Serratos, 1971). The fibres were allowed to rest for 1 hr at room temperature and tested for excitability afterwards. If they gave brief twitches and no visual sign of damage was observed, they were transferred to the experimental chamber in a small boat filled with Ringer solution. The experimental chamber, general procedure, force recording, etc. were similar to the ones described previously (Gonzalez-Serratos, 1971, 1975). The output of the force transducer was also fed in parallel to an R.C. circuit with the appropriate time constant (2 msec) to measure the rates of force development on the oscilloscope. The experiments were carried out at room temperature (19–21 °C).

Before starting the experiments four to six twitches (one per minute) were recorded, followed by two or three control tetanic stimulations at 40–50 Hz. Between each tetanus the fibres rested for at least 10 min.

Measurement of sarcomere spacings. In order to avoid variations in force development due to differences in the amount of stretching, particularly of the split fibres (Endo, 1972; Gonzalez-Serratos, Valle-Aguilera & Cillero, 1973), the overall length of the fibres was adjusted to give an average sarcomere spacing of 2·5 μm. Care was taken to maintain this average sarcomere spacing throughout the experiment. This was done by projecting the diffraction pattern produced by illuminating the fibres with a laser beam, onto a fixed screen and ensuring that the first-order diffraction pattern always fell on appropriate points on the screen.

Intracellular recordings. Whole semitendinosus muscles of the same species of frog were used to measure the membrane potentials. The muscles were stretched 1·25 × their equilibrium length (this is the length acquired by a muscle left loose in Ringer solution). This amount of stretching will give an average sarcomere length of about 2·5 μm. The recordings were done with intracellular micro-electrodes of the Ling–Gerard type with an impedance of 10–20 MΩ. The micropipettes were connected through an Ag–AgCl bridge to the input of a follower with negative capacity compensation (W.P. Instruments) and displayed on an oscilloscope (Tektronix).

Split fibres

In order to expose the interior of the muscle fibres directly to external substances, fibres split along their longitudinal axis were used. This preparation, described by Endo & Nakajima (1973), had the advantage, for this series of experiments, of exposing in a uniform way all the interior of the fibres to the external solution.

Dissection. Five to ten fibres attached to the same tendons were isolated from the semitendinosus muscle in standard Ringer solution. After 1 hr they were tested for excitability and the Ringer solution changed for relaxing solution (Table 1). Because of its high K^+ concentration this solution produced a contracture followed after a few seconds by relaxation. Immediately afterwards each of the fibres was split along the longitudinal axis as described by Endo & Nakajima (1973), except that the remaining half was left attached to the tendons. In this way the two tendons served to hold all the split fibres. After splitting, the fibres were flooded with cold relaxing solution and were stored at 0 °C until used.

The experimental arrangement. The split fibres were transferred to the experimental chamber in the same way as the intact ones, except that they were kept in the relaxing solution at 4–5 °C.

The experimental chamber was made from glass cut from microscope slides. It had a narrow channel (1·7 cm long, 0·15 cm wide and 0·15 cm deep) where the bundle was placed. The bundle was held with forceps by one tendon and the other tendon was hooked onto the force transducer (RCA 5734). The channel was covered with a glass coverslip. Since the volume of the channel was only 0·038 cm^3 the solution contained in it could be replaced several times very rapidly by injecting only 0·5 cm^3 of a solution into one end of the channel while at the same time sucking off the excess from the other end. The temperature of the chamber was controlled with a Peltier system and measured at the outlet of the channel with a thermistor.

The chamber, together with the heat sink, was set on the stage of a compound microscope. The sarcomere spacing were measured directly with the microscope as described before (Gonzalez-Serratos, 1975) and adjusted to 2·5 μm per sarcomere space.

TABLE 1. Composition of solutions used for the split fibres concentrations, (m-mole/l. H_2O. b, c and d show the free Ca^{2+} concentration (m-mole/l.)

		KMS*	Tris†	Maleic acid†	ATP-Na_2	$MgSO_4$	EGTA	$CaSO_4$	pH
R	Relaxing solution	107	20	20	4	4	2	0	6·8
L_1-b‡	5×10^{-7} Ca^{2+}	77	20	20	4	4	10	2	6·8
L_1-c	1×10^{-6} Ca^{2+}	73	20	20	4	4	10	3·33	6·8
L_1-d	1×10^{-5} Ca^{2+}	58	20	20	4	4	10	8·34	6·8

* KMS: potassium methane sulphonate.

† Tris and maleic acid were used as Tris-maleate buffer.

‡ L_2 will refer to solutions having the same composition as L_1 solutions except that cyclic AMP was added to them at the concentration indicated in the text or Figures.

Skinned fibres

In order to study the effect of adrenaline on the contraction–relaxation cycle of segments of muscle fibres where the outer membrane had been totally removed, the method of Gillis, Maes & Verellen (1973) was used. Long pieces of muscle were removed rapidly from the semitendinosus muscle of the frog, laid on a glass microscope slide and covered in light paraffin oil. The individual fibres were then gently teased apart with fine forceps and the best ones selected for 'skinning'. For good sarcomere length measurement, the fibres had to be thin, but the thinnest fibres were not sufficiently robust to give consistent results: a diameter of about 70 μm was most satisfactory. They were 'skinned' by hand; the point of a piece of fine tungsten wire was slid gently under the membrane until a split appeared which spread around the entire circumference of the fibre. The whole of the outer membrane could then be peeled back along the fibre for a distance of several hundred micrometres with little trouble. The slides carrying the fibres with their long central segments free of outer membrane were then placed in a polarizing microscope with a long-working-distance reflecting lens. A cine camera was mounted above. Local contraction of the fibre was brought about by short contact with an agar gel containing Ca^{2+} ions, which protruded from the tip of a glass micropipette, diameter 20 μm. The gel was made up to 1 % agar–agar, 0·5 % gelatine, 120 mM-KCl and 1 mM-$CaCl_2$. In order that a constant quantity of Ca^{2+} would be delivered to the muscle fibre for each contraction, the time of contact between the gel and the fibre had to be controlled. Movement of the pipette from a safe distance of 60 μm away under oil, to firm but gentle contact with the fibre was produced by passing current for a fixed time through two piezo-electric elements with a steel spring between them on which the pipette was mounted. Contact time was 650 msec. In this way controlled local contraction and relaxation cycles could be seen clearly as shortening and lengthening of sarcomere length in a small portion of the muscle. The whole sequence was filmed on Kodak 4-x film at 16 frames/sec and the sarcomere lengths were later measured and plotted against time. Control cycles of this sort are remarkably constant and repeatable, so any influence of an external agent becomes clearly apparent. In these experiments adrenaline (10^{-6} and 10^{-5} M) was stored in a second pipette and applied in small volumes to the fibres. The method for storing the adrenaline was to dip the tip of the pipette alternately in paraffin oil and in an aqueous solution of adrenaline in isosmotic KCl. This gave alternating regions of oil and adrenaline down the length of the pipette which could be photographed for estimation of their volume. Gentle application of water pressure to the wide end of the pipette discharged the drops of adrenaline singly onto the 'skinned' fibre. Any immediate reaction or subsequent alteration of the Ca^{2+}-induced contraction–relaxation cycle was then observed.

Solutions

The standard Ringer solution had the following composition (Adrian, 1956), in mmol/l.: NaCl, 115; KCl, 2·5; $CaCl_2$, 1·8; NaH_2PO_4, 0·85; Na_2HPO_4, 2·15; pH was adjusted to 7·0. The catecholamines were added in the desired concentrations as indicated in the results.

The relaxing solution (R) and loading solutions (L_1-b to L_1-d) used for the split fibres are shown in Table 1 (Endo & Nakajima, 1973; M. Endo, personal communication). For loading solutions (L_2) with N^6-2′-*O*-dibutyryl cyclic AMP (Sigma), cyclic AMP was added to the desired solution at the concentrations indicated in the Results. The releasing solution was made by adding 25 mM-caffeine (Eastman) to the relaxing solution. Special care was taken to maintain the pH of all the solutions at 6·8. All the final solutions were prepared (except ATP, which was added in its crystal form) from stock solutions immediately before the experiments and maintained close 0 °C.

RESULTS

Experiments with intact fibres

Effect of adrenaline on the twitch. The first step was to determine whether adrenaline has a clear inotropic effect in either isolated single muscle fibres or small bundles of two to four fibres. When the frogs were in good health, the results were always positive. An example of one of these experiments is illustrated in Fig. 1. Five minutes

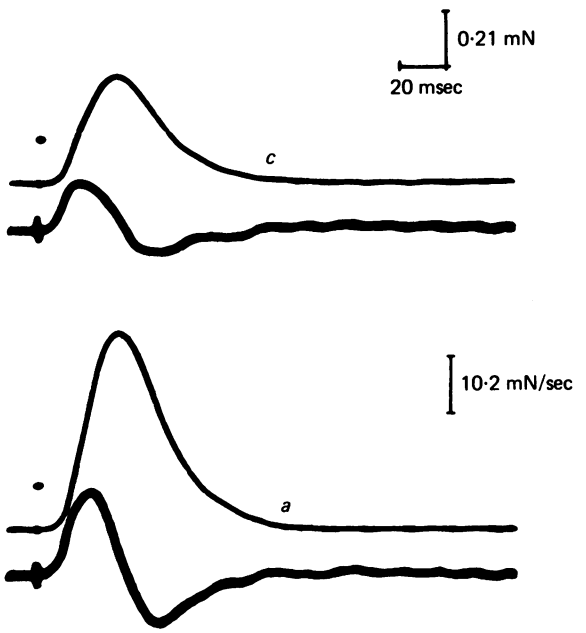

Fig. 1. Effect of L-adrenaline (1×10^{-6} M) on the twitch tension (upper traces) and rates of tension development and decay (lower traces). In *c* the fibre had not been exposed to adrenaline; in *a* the fibre had been in a bathing solution containing adrenaline for 8 min before it was stimulated. This and the experiments illustrated in the following figures were done at 20 °C unless otherwise stated. Experiment 11.IX.73.

after a control twitch (*c*) the fibre began to be bathed continuously with a Ringer solution containing 1×10^{-6} M-L-adrenaline. Eight minutes after the exposure to adrenaline started, the fibre was stimulated with a single shock again (*a*). A clear increase in twitch tension of 80 % over the control was seen under the adrenaline exposure. Although the total duration of the twitch remains approximately the same, the maximal rates of tension development and tension decay also increase by 80 % and 50 % respectively. With a Ringer solution containing 1×10^{-5} M adrenaline, the twitch tension increased up to 90 % over the control.

Time course of the positive inotropic effect. The adrenergic substances could act through a direct effect on one or more of the excitation–contraction (e–c) coupling steps or indirectly by releasing an intermediate metabolic substance from the receptor site which would eventually affect the e–c chain. If it were a direct influence, the effect produced by the adrenergic substance might appear relatively quickly, while if it were indirect, a slower time course might be expected.

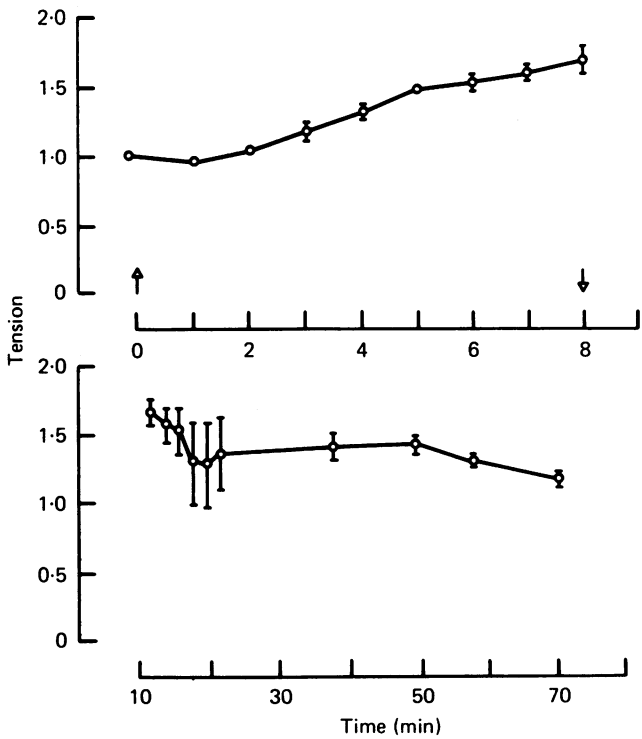

Fig. 2. Time course of the effect of 5×10^{-6} M of adrenaline on the maximal twitch tension development (top plot) and recovery (bottom plot). Tension is plotted as a fractional change of the control (1). Adrenaline was added at time 0 (first arrow) and washed out 8 min later (second arrow). During the recovery, the Ringer solution was being replaced continuously. Each circle is the average of seven observations ± the standard deviation. Experiment 6.IV.75.

The time course of the development of the increments in twitch tension due to 5×10^{-6} M adrenaline was investigated. As shown in Fig. 2, this is a relatively slow process. It took at least 3 min for the positive inotropic effect to start appearing and 8 min to reach the maximal value. As illustrated in the lower part of the figure, it took at least half an hour for the twitch tension to recover to approximately the control values after washing away the adrenaline. There is a large dispersion of the twitch tension values as the adrenaline is being washed out. We do not have a clear explanation of this effect.

Influence of adrenaline at different frequencies of stimulation. The principal features of the influence of adrenaline on the twitch are an increase of twitch tension

accompanied by an increase of the maximal rates of tension development and tension decay. One possible mechanism that could account for the above effects would be an adrenaline stimulation of the Ca^{2+} pump in the SR. If the adrenaline increases the rate of the SR Ca^{2+} pump, a resting muscle fibre exposed to adrenaline should have an increased Ca^{2+} concentration within the SR. Then during stimulation at higher frequencies there should be an increment in the rising phase of tetanic tension development due to the release of greater amounts of Ca^{2+} from the SR. After the extra Ca^{2+} accumulated in the SR has been released by a series of stimuli, the tension should approach the same level as in control contractions. The magnitude and duration of the tension increment would depend on how much of the extra Ca^{2+} in the SR is being released and how long it takes to return to normal, which will also depend on how much time there is between shocks for Ca^{2+} to be pumped back in at the higher rate. Also, since the frequency of tetanic stimulation determines the rate at which sarcoplasmic free Ca^{2+} concentration reaches saturation level for the cross-bridge reactions, an increase in the amount of Ca^{2+} released with each shock should lead to a faster rise to saturation. This effect would be more noticeable at unfused frequencies where the fibre without adrenaline is never fully saturated with Ca^{2+}. The duration of the tension increment might therefore reflect this difference in rate of rise to maximum tension, as well as the question of how long the SR can continue to release larger amounts of Ca^{2+}. For both these reasons, with a given exposure to adrenaline, at high frequencies of stimulation the duration of the extra tetanic tension would be relatively short; at unfused frequencies the extra tension should remain for a longer period; and with a train of twitches there might be enough time between shocks for extra Ca^{2+} to be pumped into the SR, giving a continuously potentiated twitch tension.

In Fig. 3 three experiments at different frequencies of stimulation are illustrated. In each one the preparation rested for 10 min after the control stimulation and then was bathed continuously with Ringer solution containing adrenaline at a concentration of 1×10^{-6} M; 7 min afterwards the preparation was stimulated again. As shown in *A*, at high frequency of stimulation (70 Hz) there was a clear increase in the tension development at the beginning of the tetanus only: eventually both tensions became similar. In this experiment, after 100 msec of stimulation, the tension developed with the fibre in adrenaline was only 1·06 times larger than the control. At a lower frequency of stimulation (40 Hz), as shown in *B*, after 100 msec of stimulation the preparations under adrenaline had developed 1·34 times the control tension and after 200 msec the tension was still 1·23 times larger. When the frequency of stimulation was sufficiently slow (3·4 Hz) to enable the individual twitches to relax completely, as shown in Fig. 3*C*, with adrenaline there was a clear potentiation of twitch tension during the entire period of stimulation. Although the peak tension decreases at the beginning it settles after a short period to a steady value, which is larger than the control. The control twitches also decline at first, as is commonly seen in muscles after a rest, but the amount by which the twitch tension decreases in adrenaline is much greater than in the control.

Effect of adrenaline on resting and action membrane potentials. The above results could perhaps be explained by sarcolemmal Ca^{2+} conductance changes induced by the adrenaline. If this were so, such a change would be reflected in the shape of the action

potential and probably also in the value of the resting membrane potential. Therefore, we explored these parameters. In eight different muscles no clear changes in the shape of the action potential were detected with 1×10^{-5} M- and 5×10^{-6} M-adrenaline concentrations. The values of the resting membrane potential and the size of the action potential are collected in Table 2. No significant difference attributable to the adrenaline was found.

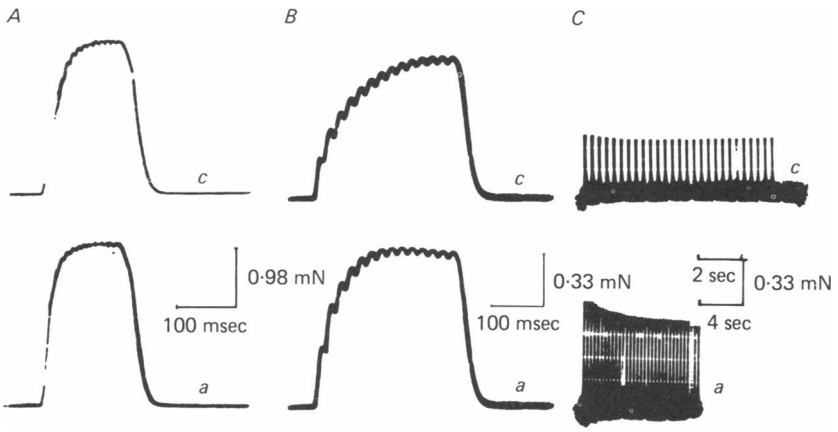

Fig. 3. Effect of adrenaline on the force development at three different frequencies of stimulation. In all the experiments adrenaline at a concentration of 1×10^{-6} M was added after the control (*c*) stimulation and a second stimulation (*a*) *was given 8 min later*. *A*, frequency of stimulation 70 Hz, experiment 6.VI.75. *B*, frequency of stimulation 40 Hz, experiment 10.VI.75. *C*, frequency of stimulation 3·4 Hz, experiment 2.VI.75. Notice in *C* the difference in time scale between the control *c* and the stimulation with adrenaline *a*.

TABLE 2. Effect of adrenaline on resting and action potentials

	RP (mV)	AP (mV)
Control	$-83 \pm 5{\cdot}6$ (40)	$116{\cdot}7 \pm 4{\cdot}6$ (40)
L-adrenaline, 1×10^{-6} M	$-88 \pm 3{\cdot}1$ (38)	$112{\cdot}0 \pm 5{\cdot}9$ (38)
Control	$-84 \pm 3{\cdot}6$ (42)	$115{\cdot}0 \pm 8{\cdot}1$ (42)
L-adrenaline, 5×10^{-6} M	$-84 \pm 5{\cdot}2$ (37)	$120{\cdot}3 \pm 10$ (37)

RP, resting membrane potential; AP, size of the action potential values are mean ± S.D. Numbers in parentheses indicate the number of observations.

Experiments in split muscle fibres

The problem was then to elucidate further the mechanism by which adrenaline causes an increase in twitch tension. It is widely accepted that adrenergic substances increase the production of cyclic AMP. This effect is mediated by the adenylatecyclase system present in the cellular membranes. Therefore, a possible mechanism by which adrenaline produces its positive inotropic effect is through the production of cyclic AMP. This nucleotide could, in turn, stimulate the SR Ca^{2+} pump. One way to explore this possibility was to expose the myoplasm to cyclic AMP directly, to see whether this increased the amount of Ca^{2+} stored within the SR. It has been reported that

the sarcolemma of skeletal muscle is not permeable to cyclic AMP (Robinson, Butcher & Sutherland, 1971), so cyclic AMP could only be given to the interior of the cell either by ionophoretic injection (Tsien, 1973) or by opening the cell and bathing it with a solution containing the substance. The second approach (see Methods) is preferable as it permits exposure of all the interior of the fibre. An experimental means of controlling Ca^{2+} release is then required. After the fibres are split the SR is left with unknown amounts of Ca^{2+}. Therefore the SR was unloaded by exposing the fibre to a relaxing solution containing 25 mM-caffeine until no tension remained (Endo & Nakajima, 1973). This was the starting condition from which the SR was reloaded by incubating the fibres for fixed periods of time in solutions containing known amounts of Ca^{2+}. After the loading period, the Ca^{2+} was again released with caffeine (25 mM). The amount of tension then produced was interpreted as being related to the amount of Ca^{2+} released from the SR.

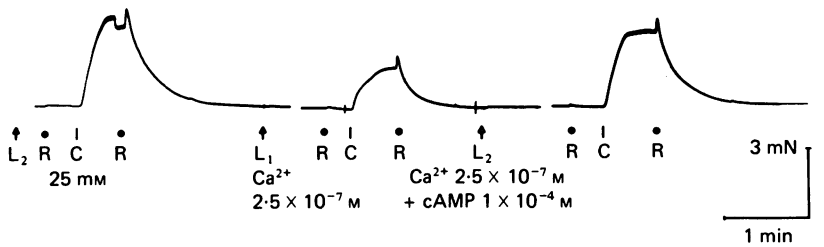

Fig. 4. Effect of the presence of cyclic AMP in the loading solution on the tension development produced by caffeine after the loading period. On the left the fibre was loaded for 20 min with a solution containing Ca^{2+} and cyclic AMP (L_2). Following the loading period the solution was washed away with a relaxing solution (R), and 20 sec later an R solution containing cafeine (C) superfused the fibre. The contracture developed in the presence of caffeine was ended by washing it away with an R soluton (R). At L_1, the fibre was loaded with a solution with no cyclic AMP. The sequence was repeated as before. The last loading period took place again in an L_2 solution (with cyclic AMP). Only the beginning and the end of each loading period is shown. In the first contracture a small drop in tension can be noticed, during the plateau: probably a split fibre broke. The first and third contractures are then similar and larger than the second one whose load took place in the absence of cyclic AMP. The concentrations of the solutions are indicated in the Figure; temperature 2·3 °C. Experiment 17.XII.76. Eight split fibres.

One of these experiments is illustrated in Fig. 4. The unloaded fibres were incubated for 20 min in a solution containing Ca^{2+} plus cyclic AMP (Solution L_2). After this loading period the L_2 solution was washed away and replaced briefly by the relaxing solution (solution R), followed by the caffeine exposure. When the development of tension reached a plateau, the preparation was bathed with the relaxing solution. When relaxation was complete the next loading period took place, lasting the same time as before but with a solution containing Ca^{2+} and no cyclic AMP (solution L_1). When caffeine was given, the amount of tension produced was around 60 % less than when the loading took place in the presence of cyclic AMP (middle record). After a third loading period with cyclic AMP present, caffeine again induced a larger tension development. The larger tension with the same amount of caffeine can be interpreted as a larger Ca^{2+} release from the SR. Since the load time and Ca^{2+} concentration were the same for all the loading periods, the fact that more tension was produced when

cyclic AMP was present strongly suggests that the nucleotide stimulates the SR Ca^{2+} pump. Nevertheless, it might be possible that cyclic AMP became bound to the contractile proteins during this rather long loading period. This could produce changes that might increase the tension development during the next Ca^{2+} release. Therefore in other experiments done at the same temperature (Fig. 5*A*) the loading time was reduced to a few minutes by increasing the Ca^{2+} concentration in the L solution to 5×10^{-7} M. The sequence was reversed so that the first loading period took

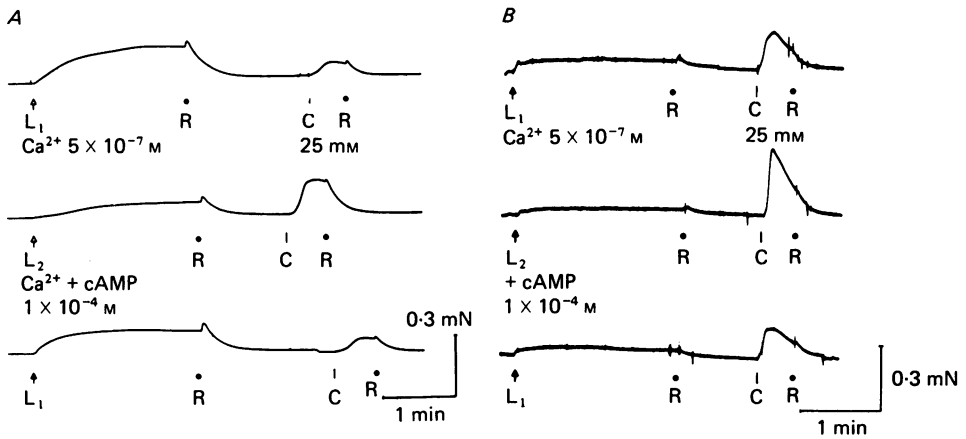

Fig. 5. Effect of cyclic AMP in the loading solution containing a higher external Ca^{2+} concentration on the tension development produced by caffeine. Top and bottom records are control and recovery runs. The fibres were loaded at L_1 with Ca^{2+}; at R they were exposed to the relaxing solution and then exposed to caffeine (C); the caffeine contracture was ended with relaxing solution at R. On the middle record the loading solution (L_2) developed during the caffeine contracture was larger when the loading period took place in the presence of cyclic AMP. All the traces shown are part of a continuous record; they were cut only for illustrative purposes. Concentrations are indicated. In *A* the experiment (7.I.77) was done at 2·4 °C with five split fibres. In *B* (experiment 18.I.77) it was done at 6 °C with three split fibres.

place without cyclic AMP (top trace), followed by the loading with cyclic AMP (middle trace) and another control loading at the end (bottom trace). During the loading time (3 min) tension developed due to a direct effect of this concentration of Ca^{2+} on the actomyosin system, but when cyclic AMP was present in the L solution the tension development due to this direct stimulation was less than when cyclic AMP was absent. Again, the tension developed during the Ca^{2+} release from the SR was larger if the Ca^{2+} uptake by the SR took place in the presence of cyclic AMP. In this case, the increase in tension after the loading with cyclic AMP was 2·17 times the control values. When the same type of experiment was done at 6 °C (Fig. 5*B*), the tension produced by direct activation with Ca^{2+} during the loading period nearly disappeared and the influence of cyclic AMP in decreasing the tension development during this period became relatively smaller. When loading was done in the presence of cyclic AMP the tension produced by the release of Ca^{2+} with caffeine increased as before around twofold; also the ratio of tension due to Ca^{2+} released by caffeine over tension due to direct activation during loading was around 2·5 times larger at 6 °C

than at 2·4 °C, while the ratio of the tension due to direct Ca^{2+} activation in the presence of cyclic AMP over the one without was only 1·6 times larger at 6 °C than at 2·4 °C. This indicates that at the higher temperature there has been a greater release of SR Ca^{2+} than at the lower temperature. If this is due to an increase in the SR Ca^{2+} content then it demonstrates the temperature-dependence of the stimulation of the SR Ca^{2+} pump by cyclic AMP.

We do not have clear proof of any explanation for the decrease in tension produced during the Ca^{2+} loading period by the presence of cyclic AMP, but one possibility

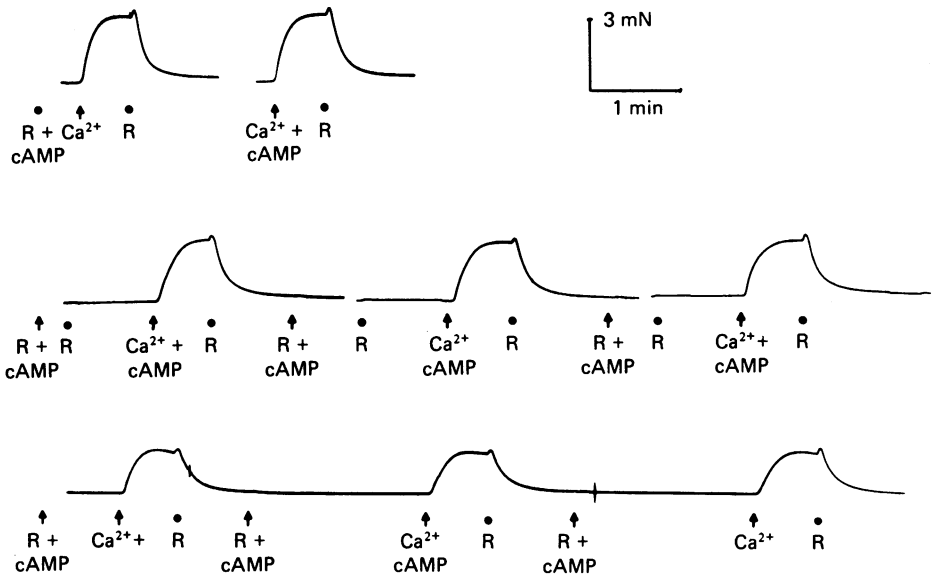

Fig. 6. Effect of cyclic AMP on the force development produced by the direct activation of Ca^{2+} on the actomyosin–ATPase system. The solutions bathing the preparation were changed as follows: in 'R' it was standard relaxing solution, in 'Ca^{2+}' it contained Ca^{2+} (1×10^{-6} M); in 'Ca^{2+} + cAMP' it contained the same amount of Ca^{2+} plus cyclic AMP (1×10^{-4} M); in 'R + cAMP' it contained cyclic AMP alone. The sequence in which the experiment was done was from top left to bottom right. The Ca^{2+} concentration was the same throughout. Temperature 6·9 °C. Experiment 2.II.77.

might be that since the Ca^{2+} concentration was below that necessary for maximal activation (Julian, 1971), the increase in the rate of Ca^{2+} pumping could effectively reduce the Ca^{2+} available for the actomyosin–ATPase system. Another possibility is that cyclic AMP interacts with the actomyosin–ATPase system directly. We do not have enough evidence to accept or reject either of these propositions. Still another possibility could be that with larger Ca^{2+} concentrations, enough to saturate the troponin Ca sites and to allow a maximal re-uptake of Ca^{2+} by the SR, the direct mechanical activation due to Ca^{2+} in the presence of cyclic AMP would be larger than without cyclic AMP. That is, that cyclic AMP might have a direct positive inotropic effect through the actomyosin–ATPase system. However, in three experiments, we investigated the tension – Ca^{2+} concentration curve with and without cyclic AMP present in the solutions. With concentrations of Ca^{2+} that produced maximal activation, the tension was the same with or without the nucleotide (Fig. 6). With

lower Ca^{2+} concentrations the tension produced with a given amount of Ca^{2+} was always less when cyclic AMP was present. McClellan & Winegrad (1978) have reported the same result in chemically skinned heart cell preparations. Further, in case the sequence of events should be critical in this respect, we produced maximal activation of the actomyosin system under different conditions of incubation with cyclic AMP (Fig. 6). It can be seen that in none of the circumstances illustrated does cyclic AMP increase the tension produced by directly activating the contractile actomyosin–ATPase system. Below saturation, as stated above and illustrated in Fig. 5*A* and *B*, cyclic AMP decreases the tension produced by direct Ca^{2+} activation.

The findings described above would indicate that cyclic AMP does not produce an increase in force development due to a direct influence on the interaction of the actomyosin–ATPase system.

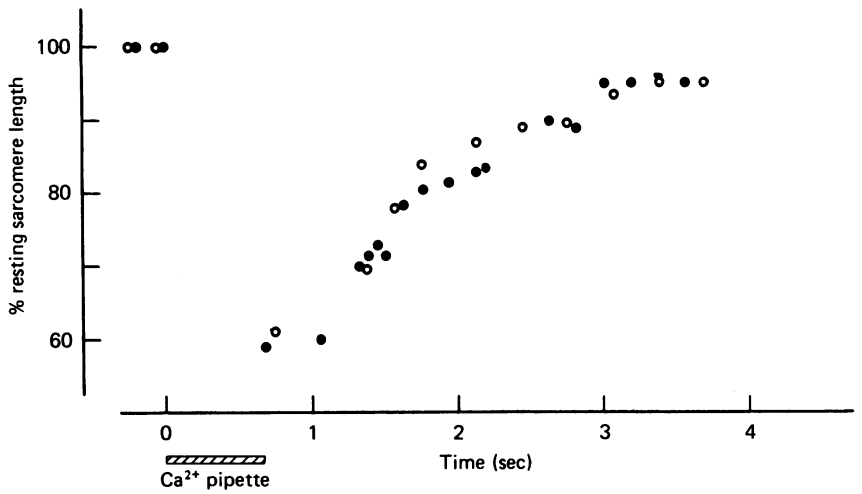

Fig. 7. Local contraction–relaxation cycles in a skinned fibre before (filled circles) and after (open circles) direct application of adrenaline (10^{-6} M).

Effect of adrenaline on skinned fibres

A further question was whether adrenaline itself rather than cyclic AMP could produce any change on the contractile activity when applied directly into the myoplasm with no sarcolemma present. This question was not only interesting in itself, but if experiments gave a positive answer it would be in contradiction to the proposition stated so far, i.e. that adrenaline acts on the SR Ca^{2+} pump through cyclic AMP, a product from the adrenergic receptor sites in the sarcolemma. The split fibre preparation was not appropriate for this type of experiment since half of the sarcolemma is left. Instead, skinned fibres were used in which the entire sarcolemma was removed, under oil, from a long central segment. Local contractions and relaxations were induced by application of Ca^{2+} from a pipette, observed under a microscope, and photographed.

Applications of adrenaline (10^{-6} and 10^{-5} M), in volumes of approximately 65×10^{-12} l. to a fibre of diameter 80 μm had no effect on the contraction–relaxation

cycle, compared with a previous control cycle. Application of a second drop of similar volume also had no effect. The time courses of contraction and relaxation before and after adrenaline are shown in Fig. 7.

Differences in time courses may have several causes: movement of the fibre relative to the Ca^{2+} pipette between test contractions; less friction between the fibre and the glass slide on which it rests because of the aqueous adrenaline solution spreading under the fibre; involvement of a distant damaged area; and of course a true drug action. However when the time courses are identical, as shown here, it is possible to be certain of the result: the adrenaline by itself has no effect.

DISCUSSION

Our results show that adrenergic substances produce a positive inotropic effect in skeletal twitch muscle fibres where the sarcolemma is present, which is characterized by increases in the twitch tension, maximum rate of tension development and rate of tension decay. With tetanic stimulation, the increase in force development associated with adrenaline appears only at the beginning of the tetanus and tends to disappear faster when higher frequencies of activation are used. When adrenaline was applied directly to the myoplasm in skinned fibres there was no change in the contractility; however, in split fibres cyclic AMP increased substantially the amount of Ca^{2+} taken up by the SR during the loading period. The above features lead us to conclude that the positive inotropic effect is due to a stimulation of the SR Ca^{2+} pump and to an increase in the capacity of Ca^{2+} binding in the SR. This leads to a larger Ca^{2+} concentration within the SR, and the release of this extra Ca^{2+} during activation is responsible for much of the increase in twitch tension. Where the extra Ca^{2+} comes from, is a matter of speculation. More and new experiments would be necessary to solve this question. But several possibilities can be mentioned: for example Ca^{2+} might recycle faster within the muscle cell, or the rate of passive Ca^{2+} influx might be slightly increased or the efflux decreased (Curtis, 1966). Adrenergic substances presumably stimulate the Ca^{2+} pump through a messenger (cyclic AMP) liberated from the receptor site located in the sarcolemma. This proposed chain of events explains the slow time course of the positive inotropic effect, which is similar to the one reported for mammalian (Brown, Bülbring & Burns, 1948; Brown *et al.* 1950; Goffart & Ritchie, 1952; Bowman & Zaimis, 1958) and frog (Oota & Nagai, 1977) skeletal muscles.

Reuter (1974) and Reuter & Scholz (1977) showed that in the mammalian heart adrenaline increases the secondary inward current carried primarily by Ca^{2+} ions, and thereby produces an increase of Ca^{2+} within the cell. Based on the delay between the increases in the secondary inward current and the contractile force, Reuter (1974) suggested that Ca^{2+} entering the fibre was used to load the internal stores from which it wold be liberated later during depolarization. Tsien (1973) demonstrated that the injection of cyclic AMP into heart cells mimics the action of catecholamines on the resting and action potentials, suggesting that cyclic AMP may act on the membrane proteins (Tsien, 1974). Furthermore, Kirchberger, Tada & Katz (1974) have shown that in isolated SR vesicles of the heart there is a phosphorylation of a cyclic-AMP-dependent proteinkinase. They proposed that the protein would be associated

with the regulation of Ca^{2+} transport into the SR. Morad & Rolett (1972), using intact myocardial cells, showed that adrenaline increases the rate of relaxation, while Fabiato & Fabiato (1975) showed a similar effect of cyclic AMP on skinned cardiac cells. Both substances presumably stimulate the uptake of Ca^{2+} ions by the SR. A reasonable scheme for the mammalian heart cells would be that adrenaline stimulates the production of cyclic AMP and that the cyclic AMP produces an increase in Ca^{2+} entry into the cell. The cyclic AMP also increases the rate of Ca^{2+} uptake into the SR, increasing the concentration of this ion within the SR and allowing the release of more Ca^{2+} during the activation. This is essentially the same system that we are proposing for skeletal muscle cells, except that adrenaline does not seem to affect the entry of Ca^{2+} ions through the sarcolemma by changing Ca^{2+} conductance. As shown in the results, and in agreement with Krnjevic & Miledi (1959), Bowman, Goldberg & Raper (1962) and Oota & Nagai (1977), adrenaline produces little change in the action and resting membrane potentials which might indicate changes in Ca^{2+} conductance. This is not surprising since changes in Ca^{2+} conductance appear to play no important role during a normal twitch in skeletal muscle (Lüttgau, 1977). Another difference between the heart and skeletal muscles is the presence in the first and absence in the second of a cyclic AMP stimulation of phosphorylation of the actomyosin–ATPase complex (Rubio *et al.* 1975; Mayer & Stull, 1971). However, they are similar in that the cyclic AMP has an inhibitory effect on the Ca^{2+} sensitivity of the activation of the contractile protein of the heart (McClellan & Winegrad, 1978) and of the skeletal muscles (this paper). Stimulation of other metabolic pumps in frog skeletal muscle by adrenaline has been reported by Hays, Dwyer & Swift (1974) and in frog skin by cyclic AMP by Aceves (1977). Oota & Nagai (1977) concluded that frog skeletal muscle has β-receptors, but unfortunately the β-blocking agent propranolol in the large concentrations they used has an inhibitory effect on the contraction itself, making the interpretation of the results very difficult indeed. Even more propranolol has local anaesthetic properties like quinidine (Goldman & Gilman, 1975).

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